Distinct TLR adjuvants differentially stimulate systemic and local innate immune responses in non-human primates

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Supplementary materials and methods

Tissue isolation

Blood samples were collected 12 days prior the injection (baseline) and at 3 and 8 hr and days 1, 2, 3, 6 and 14 post injection. Complete blood counts (CBC) were performed with total blood collected on EDTA using an automated hematology analyzer (Sysmex KX21, Mundelein, IL) for each of the samples to access the total numbers blood cells. PBMCs and plasma were isolated with the standard method from EDTA blood samples using CPT tubes (Vacutainer; BD). Plasma samples were isolated from the CPT tubes and cryopreserved for further analysis. A layer of PBMC was collected from the CPT tube, residual erythrocytes were lysed with the LCK Lysing Buffer (Lonza, Walkersville, MD) and cells were washed extensively with PBS with 5% FCS before applying for immediate antibody staining or cryopreserved in liquid nitrogen in FCS with 10% DMSO.

One inguinal lymph node was collected by biopsy at the baseline (day -12), and the left and right axillary LNs were biopsied at days 1 and 3 post injection respectively. The LNs were cleaned to remove excessive tissue. Half of the LN was collected for immunohistochemistry staining as described below. The remaining tissue was injected with 1 ml of the solution of collagenase type 4 (Worthington, Lakewood, NJ), chopped with sterile scissors, resuspended in RPMI and digested for 1 hour in 37°C. Digested LNs were further disrupted by vigorous pippetting to maximize cell release. Cells were washed in PBS/1mM EDTA and filtered twice through a cell strainer (70μm) to remove undigested tissue. Isolated LN cells were washed in PBS/1mM EDTA and stained immediately for flow cytometry analysis or cryopreserved in FCS with 10% DMSO in liquid nitrogen.

Flow Cytometry

Freshly isolated PBMC or LN cells were resuspended in PBS/1mM EDTA/5% FBS and incubated with an appropriate Ab cocktail for 30 min at 4°C. Further, cells were washed extensively with PBS/1mM EDTA/5% FBS and fixed (15 min at 4°C) using a Fixation buffer (BD). After fixing, cells were washed again and held in the PBS/1mM EDTA/5% FBS at 4°C until analysis. The following antibodies were used for surface staining of PBMC or LN cells: BD Pharmingen: CD3 APC-Cy7 (SP34-2), CD8 APC-Cy7 (SK-1), CD20 APC-Cy7 (L27), HLA-DR PE-TxRed (L243), CD123 PerCp-Cy5.5 (7G3), CD80 PE and FITC (L307.4), CD86 PE and FITC (FUN-1), CD11c APC (S-HCL-3), CCR5 PE (3A9,); Biolegend: CD14 PacBlue and ALexa-700 (M5E2), CD16 PacBlue and ALexa-700 (3G8), CD11c PE-Cy7 (3.9,); Miltenyi: BDCA-1 APC (AD5-8E7); Caltag: HLA-DR PE-TxRed (TU36) and R&D Systems: CCR7 FITC (150503).

Cytokines

Plasma cytokines were analyzed using the MILLIPLEX MAP NHP Cytokine Panel (Millipore) on a Bio-Plex 200 device (Bio-Rad). Concentrations of IFN-α (PBL Interferonsource) and IP-10 (CXCL10, R&D systems) were measured by ELISA.

Histology and immunofluorescence

Inguinal and axillary lymph nodes were collected by biopsy and snap frozen in moulds containing OCT medium, dropped into 2-methyl butane cooled with liquid nitrogen. Frozen lymph nodes were sectioned at 6μm using a Leica CM1900 UV cryostat, fixed in ice-cold acetone for 10 min, air dried and stored at −80°C. Sections were fluorescently stained with combinations of Ab: Alexa-647-labeled anti-CD20 (2H7), Alexa-488 or PE-labeled anti-CD11c (3.9, Biolegend); PE-labeled anti-CD123 (7G3, BD Pharmingen); PE-labeled anti-CD163 (RM3/1, Biolegend). Fluorescent images were captured using the ×10 and ×20 objectives on a Zeiss Axioscope (Carl Zeiss).

Statistical analysis

Statistical calculations were performed using the GraphPad Prism (La Jolla, CA). Data from all the animals in the study at day -12 (baseline) were pooled to calculate baseline

values, and compared with individual experimental groups at the indicated time-points by t-test or ANOVA.

Mass Spectrometry

R848 was quantitated by reverse phase chromatography using LC-MS/MS on an LTQ-Orbitrap mass spectrometer coupled to a Micro AS autosampler and MS pump (Thermo Scientific, Waltham, MA). Resiquimod was added to each 500uL sample and calibrator as an internal standard. The range of the assay was defined as 5nM-20uM using a six point calibration curve with all calibrators prepared in rhesus macaque serum. Samples were then prepared by acidification with trifluoroacetic acid and extracted on Oasis HLB cartridges (Waters Corporation, Milford, MA) according to the protocol described by Khier (J. Sep. Sci. 2009, 32, 1363-1373). Extracts were dried under nitrogen and resolubilized for LC-MS/MS analysis in 150uL 1:1 water/acetonitrile. LC-MS/MS analyses of R848 were performed in the Biomarkers Core Laboratory at the Yerkes National Primate Research Center at Emory University (Atlanta, GA).

TLR expression on rhesus monocytes

PBMC were isolated from blood using CPT tube as described above. PBMCs were depleted of T and B cells using anti-CD3 and anti-CD20 MACS-Beads (Miltenyi) and MACS columns (Miltenyi) according to the manufacturers protocol. The $CD20^{neg}CD3^{neg}$ cells were stained with the following Ab cocktail: CD14, CD16, HLA-DR and CD3, CD8, CD20 using the same Ab clones as in flow cytometry analysis (M&M Flow Cytometry section). The SSC-A^{hi} FSC-A^{hi} $CD3^{neg}CD8^{neg}CD20^{neg}$ HLA-DR^{+} population was further divided into 3 distinct populations by CD14 and CD16 staining: $CD14^{+}CD16^{neg}$, $CD14^{+}CD16^{+}$ and $CD14^{dim}CD16^{++}$ as described in Figure 2. Cells were sorted on a FACS Aria machine (BD). At least 5×10^{5} cells from each of the indicated monocyte sub-population were collected with a purity of 95% or higher. Sorted cells were lysed in 1 ml of TRIzol (Invitrogen) and cryopreserved at -80°C. The samples were thawed, and the RNA isolation proceeded according to the manufacturer's protocol. Total RNA sample quality was evaluated by spectrophotometer and RNA was reverse-transcribed using a SuperScript III First Strand Synthesis SystemKit Protocol

(Invitrogen). The Quantitative Real-Time PCR was performed using RT^2 Profiler PCR Array for specific for Rhesus Macaque TLR Signaling Pathway (QIAGEN). All sequences were amplified using the Bio-Rad MyIQ System. Gene expression data were normalized relative to geometric mean of two housekeeping genes (*GAPDH and B2M*).

Table S1. Custom designed Quantitative Real-Time PCR Low Density Array.

#	Gene name	Assay ID	#	Gene name	Assay ID	#	Gene name	Assay ID
1	18S	Hs99999901_s1	40	IFNGR1	Rh02787824_m1	79	PTGS2	Rh01573476_m1
2	ACTB	Rh03043379_gH	41	IFNGR2	Rh02794058_m1	80	PTPRC	Hs00365634_g1
3	BAX	Hs00180269_m1	42	IKBKB	Rh02621738_m1	81	PTX1	Rh02872128_m1
4	BCL2	Rh02848299_m1	43	IL10	Rh02789325_m1	82	PTX3	Rh03467107_m1
5	BCL2L1	Hs00169141_m1	44	IL-12A	Rh02621733_m1	83	SELE	Rh02621708_m1
6	C1QB	Rh02902677_s1	45	IL12B	Rh02621748_m1	84	SELP	Rh02621756_m1
7	C3	Rh02621703_m1	46	IL13	Rh03043053_m1	85	SKI	Rh02621757_g1
8	C3AR1	Rh02914877_m1	47	IL15	Rh02621777_m1	86	SLC2A6	Rh01115483_m1
9	CCL19	Rh02621767_m1	48	IL15RA	Rh00542602_m1	87	SMAD3	Rh02621765_m1
10	CCL3	Rh02788104_gH	49	IL17A	Rh02621750_m1	88	SMAD7	Rh00998191_m1
11	CCL4L1	Rh03063175_g1	50	IL18	Rh02787952_m1	89	STAT3	Rh01047578_mH
12	CCL5	Rh02788105_m1	51	IL1A	Rh02789772_m1	90	TBX21	Rh02621772_m1
13	CCR2B	Rh02621725_s1	52	IL1B	Rh02621711_m1	91	TGFB1	Rh02621726_m1
14	CCR4	Rh02621764_u1	53	IL2	Rh02621714_m1	92	TNFA	Rh02789784_m1
15	CCR5	Rh03043152_s1	54	IL23A	Rh02872166_m1	93	TNFRSF17	Rh02837830_m1
16	CCR7	Hs99999080_m1	55	IL2RA	Rh02788820_m1	94	TNFRSF18	Rh02847833_m1
17	CD38	Rh01120072_m1	56	IL3	Rh02621715_u1	95	TNFRSF1B	Rh02837327_m1
18	CD40LG	Rh02621704_m1	57	IL4	Rh02621716_m1	96	VEGFA	Rh02621759_m1
19	CSF1	Rh02621778_m1	58	IL5	Hs00174200_m1			
20	CSF3	Rh02621779_m1	59	IL6	Rh02621719_u1			
21	CTLA-4	Rh02787987_m1	60	IL7	Rh02621732_m1			
22	CXCL10	Rh02788358_m1	61	IL8	Rh02621713_m1			
23	CXCL11	Rh02621763_m1	62	IL9	Rh02621717_m1			
24	CXCR3	Rh02621737_s1	63	IRF1	Rh00971962_m1			
25	EDN1	Rh02621744_m1	64	IRF5	Hs00158114_m1			
26	EIF2AK4	Hs01010957_m1	65	IRF7	Rh02839174_m1			
27	FAS	Rh02787979_m1	66	ISG15	Rh02915441_g1			

28	FASLG	Rh02621724_m1	67	JUN	Rh02913397_s1			
29	FN1	Rh02621780_m1	68	JUNB	Rh02913427_g1			
30	GM-CSF	Rh02621728_m1	69	JUND	Rh02897913_s1			
31	GUSB	Rh02788764_m1	70	KBTBD7	Rh02821301_s1			
32	HMOX1	Rh02621747_m1	71	LTA	Rh02829188_g1			
33	ICAM1	Rh02621706_m1	72	MCP-1	Rh02621753_m1			
34	ICOS	Rh02621771_m1	73	MX1	Rh02842279_m1			
35	IFIT1	Rh00929909_m1	74	MYD88	Rh01573837_m1			
36	IFNA1	Hs00256882_s1	75	NFKB2	Rh02621752_m1			
37	IFNA2	Rh02902794_s1	76	NOS2	Rh02829281_m1			
38	IFNB1	Rh02913347_s1	77	OAS1	Rh00973637_m1			
39	IFNG	Rh02788577_m1	78	PITX2	Hs01553178_g1			

Supplementary figure legends

Figure S1. Expansion of white blood cells, neutrophils and monocytes mediated by injection of TLR-L. (A) Kinetics of total numbers of white blood cells (WBC) were assessed by hematological analysis and presented as a total number of cells/µl blood at the indicated time points. Statistical analysis of cell kinetics indicates significant change relevant to the baseline, mean ± SEM, t-test *p<0.05, **p<0.01, ***p<0.001. (B) Comparison of total numbers of neutrophils (red bars, top panel) and PBMC (blue bars, bottom panel) at 3 hours (open bars) and 8 hours (solid bars) post injection. Mean ± SEM, ANOVA, *p<0.05, **p<0.01, ***p<0.001.

Figure S2. R-848 and CpG-ODN induce activation of systemic pDC and mDC. (A) Gating strategy for rhesus DC populations: plasmacytoid DCs (pDC) were defined within the singlet population of freshly isolated PBMC as the lineage (Lin.: CD3, CD8, CD20, CD14, CD16)^{neg}, HLA-DR^{+} CD123^{+} cells. Myeloid DC (mDC) were identified as the Lin^{neg}, HLA-DR^{+} CD11c^{+} BDCA-1^{+} cells. Numbers in cell gates indicate frequencies of gated cells within the parent population from one representative animal at baseline (day -12). Kinetics of pDC (B) and mDC (C) after TLR-L injection were assessed by flow cytometry as indicated in (A) and are represented as cell frequencies in PBMC at the indicated time points. Statistical analysis shows significant change relevant to the baseline, mean ± SEM, t-test, *p<0.05, **p<0.01, ***p<0.001. Activation and maturation phenotypes of blood pDC (B) and mDC (C) are represented as histograms of surface CD80, CD86 and CCR7. Histograms show the isotype control (gray) and the color-coded activation phenotypes at the indicated time points post TLR-L injection. Histogram data from one representative animal are shown for each experimental group.

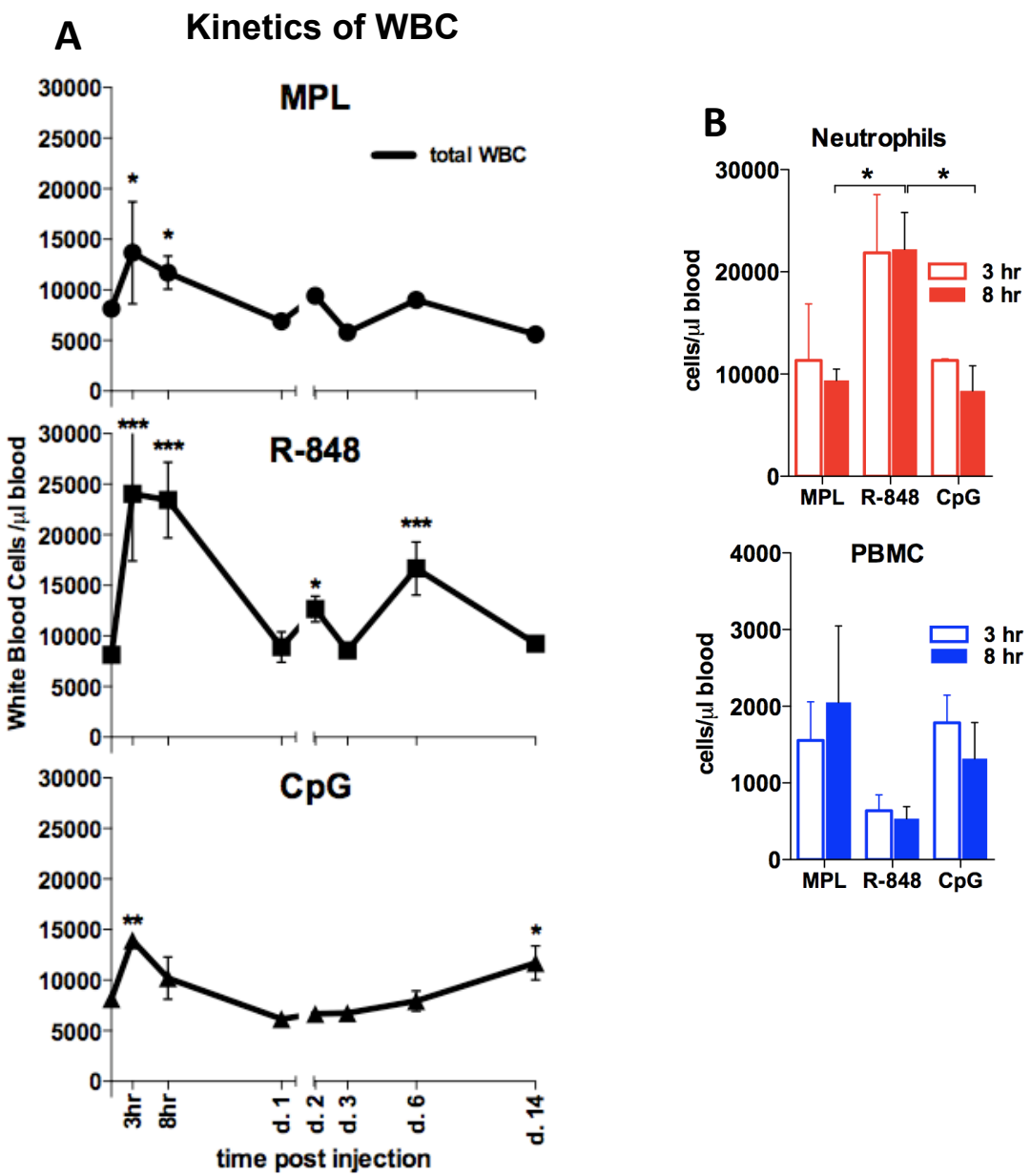
Figure S3. Immunohistochemistry staining of monocytes in LN.
Immunohistochemistry staining was performed on frozen LN sections for evaluating the in-situ localization of monocytes. Monocyte and macrophage populations were assessed in animals injected with MPL (day 3) and CpG-ODN (day 1) by CD163 (red) and CD11c

(green) staining. B cell follicles were visualized by CD20 staining (blue). One representative animal from MPL and CpG-ODN experimental groups is shown.

Figure S4. Expression of TLRs in rhesus monocyte sub-populations. Expression of TLR 1-10 was determined in the FACS-sorted sub-populations of blood monocytes: $CD14^{+}CD16^{neg}$, $CD14^{+}CD16^{+}$ and $CD14^{dim}CD16^{++}$ and whole PBMC. Relative expression was measured by quantitative RT-PCR. Values indicate the mRNA expression of indicated TLR gene relative to housekeeping gene GAPDH (glyceraldehyde phosphate dehydrogenase) and B2M (Beta-2 microglobulin). Bar graphs represent data from 2 individual rhesus animals.

Figure S5. Concentration of R-848 compound in plasma. Plasma samples were collected post R-848 injection and cryopreserved. Concentration of resiquimod was measured by mass spectrometry at the indicated time points. Mean ± SEM, t-test, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Figure S1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R-848
CpG
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20000
15000
10000
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3hr
8hr
d. 1
d. 2
d. 3
d. 6
d. 14
time post injection
B
Neutrophils
cells/μl blood
3 hr
8 hr
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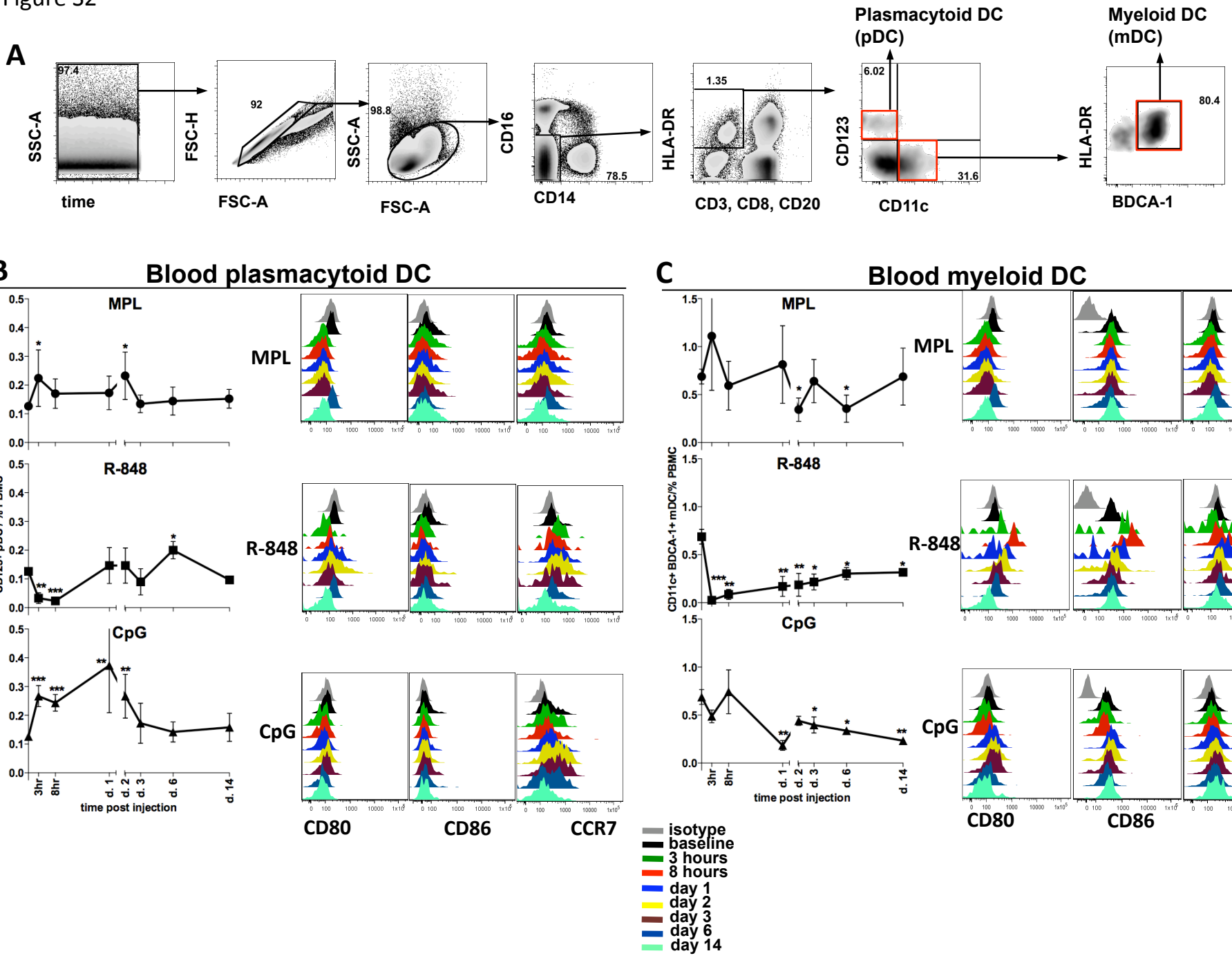
R-848
CpG
PBMC
4000
3000
2000
1000

Figure S2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Myeloid DC (mDC)
SSC-A
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SSC-A
FSC-A
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CD14
HLA-DR
CD3, CD8, CD20
CD123
CD11c
HLA-DR
BDCA-1
B
Blood plasmacytoid DC
MPL
R-848
CpG
CD123+ pDC / % PBMC
time post injection
CD80
CD86
CCR7
C
Blood myeloid DC
CD11c+ BDCA-1+ mDC/% PBMC
isotype
baseline
3 hours
8 hours
day 1
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day 3
day 6
day 14

Figure S3

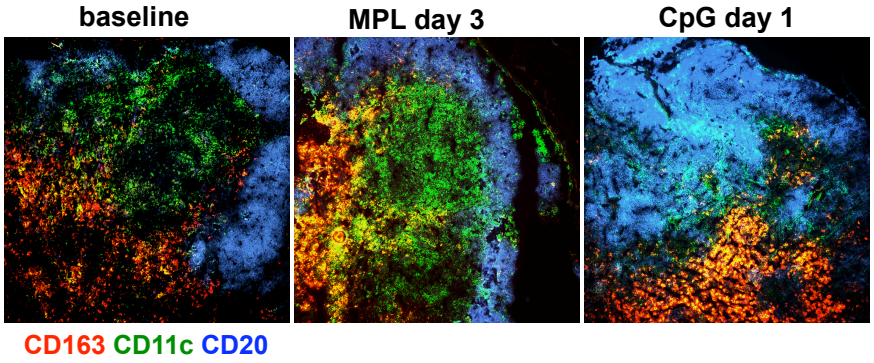
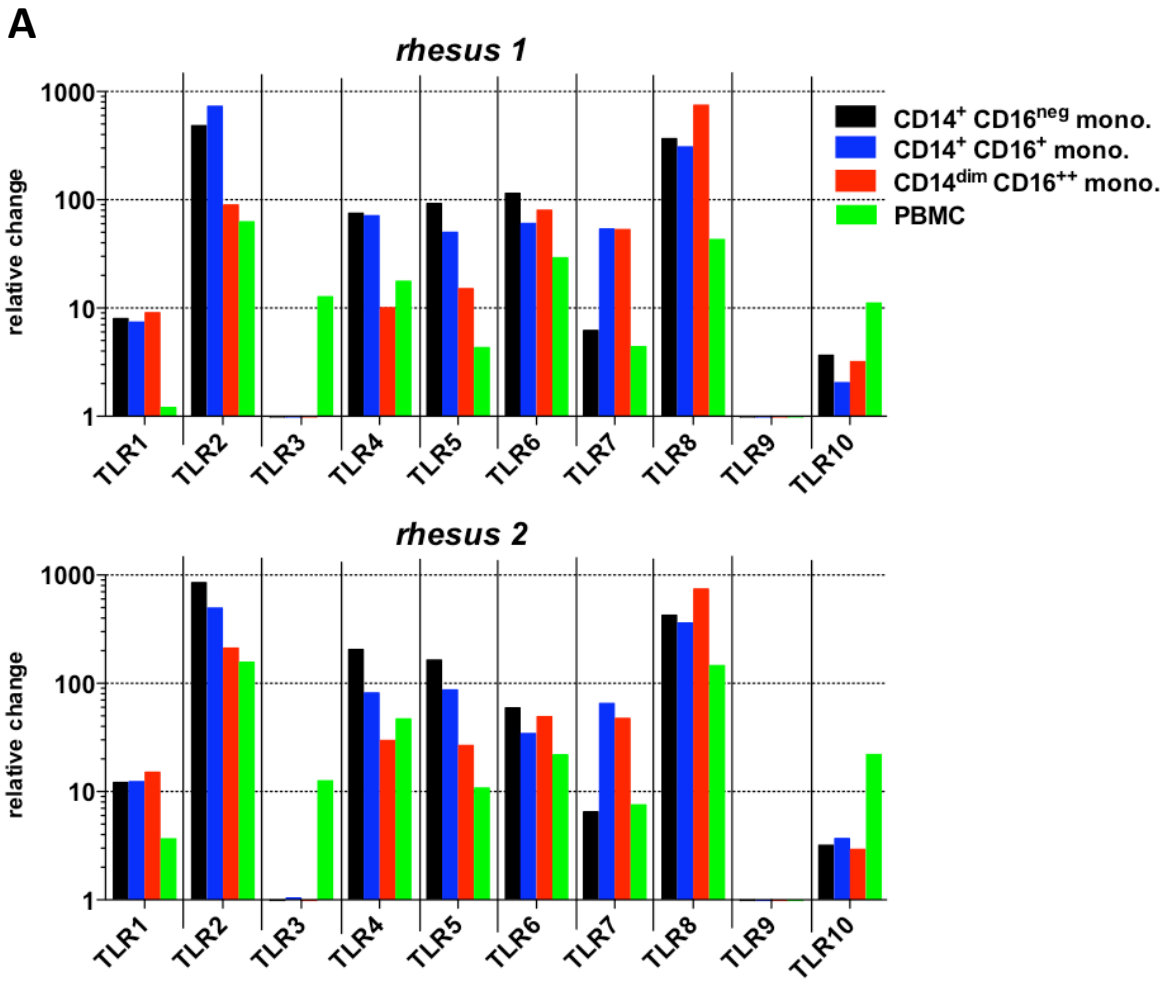


Figure S4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rhesus 1
relative change
1000
100
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1
TLR1
TLR2
TLR3
TLR4
TLR5
TLR6
TLR7
TLR8
TLR9
TLR10
CD14+ CD16neg mono.
CD14+ CD16+ mono.
CD14dim CD16++ mono.
PBMC
rhesus 2
relative change
1000
100
10
1
TLR1
TLR2
TLR3
TLR4
TLR5
TLR6
TLR7
TLR8
TLR9
TLR10

Figure S5